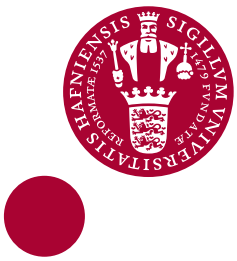

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Linkage Between Dinitrogen Fixation and Primary Production in the Oligotrophic South Pacific Ocean

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Key Points:
- Diazotroph community structure would be critical for the direct linkage between dinitrogen fixation and primary production
- Dinitrogen and carbon fixation by *Trichodesmium* contribute to primary production
- Dinitrogen fixation by UCYN-A1 and heterotrophic bacteria contributes little to fueling primary produciton

Supporting Information:
- Supporting Information S1
- Data Set S1
- Data Set S2

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Abstract The import of nitrogen via dinitrogen fixation supports primary production, particularly in the oligotrophic ocean; however, to what extent dinitrogen fixation influences primary production, and the role of specific types of diazotrophs, remains poorly understood. We examined the relationship between primary production and dinitrogen fixation together with diazotroph community structure in the oligotrophic western and eastern South Pacific Ocean and found that dinitrogen fixation was higher than nitrate-based new production. Primary production increased in the middle of the western subtropical region, where the cyanobacterium *Trichodesmium* dominated the diazotroph community and accounted for up to 7.8% of the phytoplankton community, and the abundance of other phytoplankton taxa (especially *Prochlorococcus*) was high. These results suggest that regenerated production was enhanced by nitrogen released from *Trichodesmium* and that carbon fixation by *Trichodesmium* also contributed significantly to total primary production. Although volumetric dinitrogen fixation was comparable between the western and eastern subtropical regions, primary production in the western waters was more than twice as high as that in the eastern waters, where UCYN-A1 (photoheterotroph) and heterotrophic bacteria were the dominant diazotrophs. This suggests that dinitrogen fixed by these diazotrophs contributed relatively little to primary production of the wider community, and there was limited carbon fixation by these diazotrophs. Hence, we document how the community composition of diazotrophs in the field can be reflected in how much nitrogen becomes available to the wider phytoplankton community and in how much autotrophic diazotrophs themselves fix carbon and thereby influences the magnitude of local primary production.

1. Introduction

Marine primary production is generally limited by nitrogen availability and is divided into new and regenerated production depending on the nitrogen source (Dugdale & Goering, 1967). New production is defined as the production based on nitrogenous nutrients newly entering the euphotic zone and is in a steady state system considered to be in balance with material export from the euphotic zone (Eppley & Peterson, 1979). Although Dugdale and Goering (1967) mentioned that dinitrogen fixation could contribute to new production, nitrate supply from deep waters to the euphotic zone had long been considered the major source for new production (Chavez & Toggweiler, 1995; McCarthy & Carpenter, 1983). However, dinitrogen fixation was later recognized to also support new production significantly and sometimes exceed nitrate-based new production on short time scales, in particular in tropical and subtropical oligotrophic regions where vertical nitrate supply is limited by strong thermohaline stratification (Capone et al., 2005; Karl et al., 1997).

New nitrate input from deep waters is known to enhance primary production in the oligotrophic ocean, for instance, by turbulent diffusion (Lewis et al., 1986), wind-induced vertical mixing (Babin et al., 2004; Lin et al., 2003), upwelling in the wake of islands (Furuya et al., 1986; Hasegawa et al., 2009), vertical water

displacement by mesoscale eddies (McGillicuddy et al., 1998; Johnson et al., 2010), internal waves (Sharples et al., 2001), and planetary waves (Uz et al., 2001). In comparison, the relationship between dinitrogen fixation and primary production is less clear. Satellite data indicated that algal blooms northeast of the Hawaii islands and western South Pacific were triggered by diazotrophy (Willson & Qiu, 2008). Similarly, in the same regions, increased levels of chlorophyll *a* (chl *a*) were observed to accompany blooms of *Trichodesmium* and diazotroph-diatom association (DDA; Dore et al., 2008; Villareal et al., 2012), and high primary production was associated with dinitrogen fixation by *Trichodesmium* (Shiozaki, Kodama, and Furuya, 2014). Furthermore, in the subtropical Atlantic, enhanced primary production with blooms of *Trichodesmium* and DDA were reported (Carpenter et al., 1999, 2004). These findings indicate that new nitrogen input through dinitrogen fixation enhances primary production. However, accumulating evidence shows that this is not always the case. For instance, both in the subtropical North and South Pacific gyre, where diazotrophs are commonly dominated by unicellular cyanobacteria or heterotrophic bacteria (Church et al., 2008; Halm et al., 2012; Shiozaki et al., 2017; Turk-Kubo et al., 2014), enhanced primary production appears rarely linked to dinitrogen fixation (e.g., Raimbault & Garcia, 2008; Shiozaki et al., 2017; Willson & Qiu, 2008).

In the present study we hypothesized that diazotroph community structure is critical for the direct linkage between dinitrogen fixation and primary production. This relation could involve the fate of fixed dinitrogen and the ecology and carbon fixation by diazotrophs themselves. The fate of recently fixed dinitrogen is thought to strongly depend on the diazotroph species present in the water column (Bonnet, Berthelot, Turk-Kubo, Fawcett, et al., 2016; Mulholland, 2007). *Trichodesmium* is positively buoyant, and although it can under certain conditions be found in sediment traps (Pabortsava et al., 2017), large shares of the fixed dinitrogen get recycled within the surface ocean after exudation or cell lysis and directly fuel primary production by other algae there (Hewson et al., 2004; Mulholland, 2007; O'Neil & Roman, 1992). Although our knowledge on grazing on diazotrophs is limited to that by mesosized and macrosized zooplankton (Conroy et al., 2017; Hunt et al., 2016; Scavotto et al., 2015), unicellular cyanobacteria and heterotroph diazotroph are assumed to be rapidly consumed by protists and thereby fuel the microbial food web within the euphotic zone (Caron et al., 1991). Meanwhile, unicellular diazotroph UCYN-C is known to aggregate during blooms and contribute significantly to vertical export (Bonnet, Berthelot, Turk-Kubo, Fawcett, et al., 2016; Knapp, Fawcett, et al., 2016). DDAs tend to have high sinking rates and thereby quickly transfer fixed dinitrogen to the deep sea (Scharek et al., 1999; Subramaniam et al., 2008). It is also noteworthy that when autotrophic diazotrophs account for a large portion of the phytoplankton, their carbon fixation is recognized to contribute significantly to total primary production (Carpenter et al., 2004). For most uncultured and/or recently discovered diazotrophs, however, the extent to which they directly fuel primary production in the euphotic zone is unknown.

In the present study, we examined diazotrophy in the eastern and western South Pacific. *Trichodesmium* blooms often occur in the western subtropical South Pacific, while they are rare in the eastern area (in the sense of Figure 8 of Shiozaki et al., 2010), in which the diazotroph community is known to mainly consist of heterotrophic bacteria (Halm et al., 2012; Turk-Kubo et al., 2014). Thus, our sampling enabled us to simultaneously examine the relationship between primary production, dinitrogen fixation, nitrate assimilation, and diazotroph community structure in regions with contrasting diazotroph communities and where new production was mainly controlled by dinitrogen fixation.

2. Materials and Methods

2.1. Water Sampling

Sampling was carried out on board the R/V *Hakuho-Maru* from 22 December 2013 to 13 January 2014 (KH-13-7) in the western South Pacific and from 1 to 20 January 2012 (KH-11-10) in the eastern South Pacific (Figure 1). A total of 8 and 11 stations were sampled along the ~6,500 and ~4,500 km transects in the western and eastern South Pacific, respectively. Hydrographic data were obtained using a SBE 911 plus conductivity-temperature-depth (CTD) system (Sea-Bird Electronics). Water samples were collected by an acid-cleaned bucket from the surface and by acid-cleaned Teflon-coated 12 L Niskin-X bottles on a CTD-carousel system attached to a titanium armored cable (8 mm o.d.) from other depths. Samples for incubation experiments and DNA analyses were collected from depths corresponding to 100%, 25%, 10%, 1%, and 0.1% of surface light intensity. The depth profiles of light intensity were determined using a Hyper Profiler

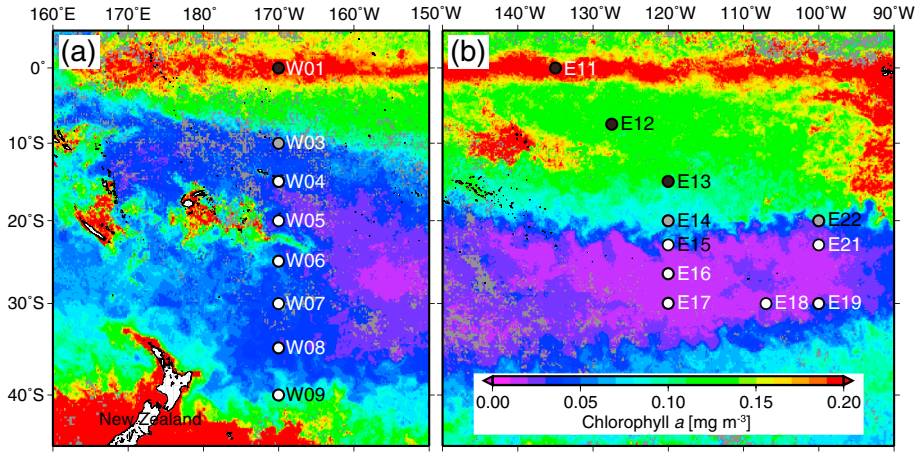

Figure 1. Stations sampled in the (a) western and (b) eastern South Pacific. Background contours denote satellite-derived (MODIS-Aqua) chlorophyll *a* concentration, composited during each observation period: A: 22 December 2013–13 January 2014; b: 1–20 January 2012. The black, gray, and white circle stations were in equatorial upwelling region, frontal region, and subtropical oligotrophic region, respectively.

(Satlantic LP, Halifax, NS, Canada). Samples for RNA analysis were only collected from surface waters in the western South Pacific. Samples for nutrients, chl *a*, and flow cytometry were collected from 5 to 12 depths within the upper 200 m. Samples for microscopy were collected from surface water. Samples for dissolved iron concentration determination were collected from 10 m and the 25% light depth in the western and eastern South Pacific, respectively, using precleaned Teflon-coated Niskin-X samplers where existing O-rings and spigots of which were replaced with Viton O-rings and Teflon spigots. This system has been successfully applied for trace metal sampling (e.g., Kim et al., 2015).

2.2. Nutrients and Dissolved Organic Nitrogen

Nitrate, nitrite, ammonium, and phosphate concentrations were immediately determined on board by supersensitive colorimetric systems with long capillary cells (Hashihama et al., 2009, 2015). Detection limits for nitrate, nitrite, and phosphate were 3, 2, and 3 nM, respectively. For the analysis of ammonium concentrations, we used systems with a 200-cm path length (UltraPath system) and a 100-cm path length liquid waveguide capillary cell in the western and eastern cruises, respectively. Detection limits of ammonium in the western and eastern cruises were 4 and 6 nM, respectively. For samples in which the nutrient concentrations were higher than 1 μM, concentrations were redetermined using an AACS II autoanalyzer onshore.

Samples for total dissolved nitrogen (TDN) analysis were filtered through a precombusted Whatman GF/F filter by gravity filtration. The filtrate was immediately frozen for later analysis. The concentration of TDN was determined by a persulfate oxidation method (Hansen & Koroleff, 1999) using an automated flow analytical system (QuAAtro, SEAL) according to manufacturer instructions. A 3% NaCl solution was used as the blank and standard matrix. The precision of the TDN analysis was ±1% at the 20 μM level. The concentration of dissolved organic nitrogen (DON) was determined by subtracting the dissolved inorganic nitrogen (DIN; nitrate + nitrite + ammonium) concentration from TDN concentration.

2.3. Dissolved Iron

Water samples were transferred directly from the Niskin-X bottle to a trace-metal-clean (Kondo et al., 2012) 125-mL low-density polyethylene bottle through a 0.2 μm Acro Pac 200 capsule filter cartridge both in the western and eastern South Pacific. The samples were then acidified (pH ~1.7) with ultrapure 20% hydrochloric acid (TAMAPURE-AA-100, Tama Chemicals Co., Ltd) and stored at room temperature until analysis.

Dissolved iron concentrations in the western South Pacific were determined using a high-resolution inductively coupled plasma-mass spectrometer (Finnigan ELEMENT2, Thermo Electron Co.) following protocols of Lagerström et al. (2013). Dissolved iron concentrations in the eastern South Pacific were determined by catalytic stripping voltammetry (Obata & van den Berg, 2001) with substitution of the pH buffer HEPPS

(3-[4-(2-hydroxyethyl)piperazin-1-yl]propane-1-sulfonic acid) by POPSO (piperazine-N,N′-bis (2-hydroxypropanesulfonic acid); Sato et al., 2007). The detection limits were 0.056 and 0.020–0.12 nM in the western and eastern South Pacific cruises, respectively.

2.4. Chlorophyll *a*, Microscopy, and Flow Cytometric Analysis

Samples for chl *a* of 0.3 L were filtered onto 25-mm Whatman GF/F filters and extracted with *N*,*N*-dimethylformamide (Suzuki & Ishimaru, 1990). Concentrations were determined using a 10-AU fluorometer (Turner Designs, Inc. San Jose, CA, USA) calibrated with a chl *a* standard (DHI, Denmark).

Samples for microscopy were collected in 500-mL polypropylene bottles, fixed with acidified Lugol's solution, and kept in the dark until analysis. The samples were quantified by the Utermöhl method using an inverted microscope. Phytoplankton were identified at species or genus level (Fiona & Harvey, 2005; Kraberg et al., 2010; Tomas, 1997), and cell volumes were calculated from assigned geometric shapes (Hillebrand et al., 1999). Since phytoplankton cells shrink due to Lugol's fixation, the cell volumes of fixed cells were multiplied with 1.33 (Montagnes et al., 1994).

Picophytoplankton and nanophytoplankton were quantified by flow cytometry (PAS-III for the western South Pacific samples, and CyFlow Space for eastern South Pacific samples; both Partec, Germany). The samples were immediately quantified on board without any chemical fixation. The mean cell diameters of eukaryotic phytoplankton and nanosized cyanobacteria were estimated using the mean value of their forward light scatter normalized to that of 2-μm polystyrene fluorescent beads (Polysciences, USA). The relationship between particle diameter and forward light scatter was obtained from measurements of 0.5-, 0.75-, 1-, 2-, 3.2-, and 6-μm beads (Polysciences, USA). For *Prochlorococcus* and *Synechococcus*, fixed diameters of 0.6 and 0.9 μm were used to calculate cell volume (Morel et al., 1993).

2.5. Nitrate Assimilation, Vertical Nitrate Flux, Dinitrogen Fixation, and Primary Production

The initial ^{15}N and ^{13}C enrichment of particulate organic matter (4 L) were collected from each light depth and were immediately filtered at the beginning of the incubation at every station.

Samples for the nitrate assimilation assays were filled into acid-cleaned 2-L polycarbonate (PC) bottles covered with neutral-density screen to adjust the light intensity at each sampling depth. Except for in the equatorial upwelling region (Station [St.] E11, E12, E13, and W01), nitrate assimilation was estimated by the Michaelis-Menten kinetics approach to correct for the overestimation caused by the excessive use of the ^{15}N tracer in nitrate-deplete waters (Shiozaki et al., 2009). ^{15}N-labeled nitrate (99 atom% ^{15}N; SI Science) was added to a final tracer concentration of 10, 20, 100, and 2,000 nM. In the equatorial upwelling region, enrichment of the ^{15}N-labeled nitrate was less than 10% of the ambient nitrate concentration. We performed short time incubation (~2 hr, supporting information Data Set S1) in an on-deck incubator at midday according to the standard protocol of Joint Global Ocean Flux Study (United Nations Educational, Scientific and Cultural Organization, 1994). At stations W01, W03, W05, W07, W08, W09, E11, E15, E18, and E21, samples collected and incubated (~2 hr) at midnight as well were used for extrapolating to daily nitrate assimilation We did not determine nitrification, but nitrification rarely occurs above the 1% light depth in subtropical oligotrophic waters (Shiozaki et al., 2016). The short 2 hr incubation periods may introduce a bias as it may cover particularly active (or inactive) phases of nitrate assimilation, by any particular microbial community at a given time. Therefore, we also compare our rate estimates to estimates of vertical nitrate flux.

Vertical nitrate fluxes were calculated from vertical eddy diffusivities and the observed gradient of nitrate concentrations at the nitracline. The vertical eddy diffusivity was assumed to be constant at 0.11 $m^2\ s^{-1}$, which is commonly assumed for oligotrophic open oceans (Capone et al., 2005). Areal rates of dinitrogen fixation, primary production, and nitrate assimilation were calculated from the surface down to the 1% light depth by trapezoidal integration, since dinitrogen fixation at the 0.1% light depth was low or undetectable in most cases, and the maximum of dinitrogen fixation and primary production was always located near the surface (see below).

Samples for dinitrogen fixation and primary production were immediately transferred into duplicate acid-washed 4.5-L PC bottles with thermoplastic elastomer caps. Dinitrogen fixation was determined by the $^{15}N_2$ gas bubble method (Montoya et al., 1996) in the eastern South Pacific, which potentially underestimates dinitrogen fixation (Mohr et al., 2010). In contrast, in the western South Pacific, dinitrogen fixation was

determined by the $^{15}N_2$ gas dissolution method (Mohr et al., 2010). $^{15}N_2$ gas supplied by SI Science was used both cruises in the western and eastern South Pacific. In the eastern cruise, 2 mL of $^{15}N_2$ gas was injected using a gas-tight syringe. In the western cruise, 100 ml of $^{15}N_2$-dissolved water, which was made from filtered seawater collected from the surface at the same station, was added (cf. Shiozaki, et al., 2015). The volume of dissolved N_2 was calculated using the equation given by Weiss (1970), and we assumed that added $^{15}N_2$ was completely dissolved. ^{13}C-labeled sodium bicarbonate (99 atom% ^{13}C; Cambridge Isotope Laboratories) was added at a final concentration of 200 μM to the same replicate bottles. Light levels were adjusted using neutral-density screens wrapped around each bottle. All bottles were incubated for ~24 hr (Data Set S1) in an on-deck incubator with flowing surface seawater. Subsequent analyses and calculations were performed as described previously (Shiozaki et al., 2009).

2.6. DNA and RNA Collection, *nifH* Amplicon Sequencing, and Sequence Analysis

Samples for DNA analyses (1–2 L) were filtered onto a 0.22-μm pore size Sterivex-GP filters (Millipore, Bedford, MA, USA) and 0.2-μm pore size Nuclepore PC membrane filters (Whatman, Kent, UK) in the western and eastern South Pacific, respectively. RNA samples (1–2 L) were collected only from surface waters in the western South Pacific during daytime and were filtered within 30 min of sampling. The filters were soaked in RNAlater (Life Technologies, Carlsbad, USA) and frozen at −80°C until RNA extractions (Shiozaki et al., 2016). Complementary DNA synthesis was made using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) following the manufacturer's guidelines and using with the nifH3 as the reverse primer (Turk et al., 2011).

Partial *nifH* fragments were amplified by a nested polymerase chain reaction (PCR) approach (Zehr & Turner, 2001) as described previously (Bentzon-Tilia et al., 2015; Shiozaki et al., 2017). Samples from the surface and from the 25% and 1% light depths were selected from stations in the western (W05–W08) and eastern (E15–E19) subtropical South Pacific with relatively high rates of dinitrogen fixation. In addition, RNA samples collected from the surface in the western subtropical South Pacific (W05–W08) were analyzed. PCR amplification, product purification, and quantification were done as previously reported (Shiozaki et al., 2017). Products were sequenced on an Illumina Miseq platform at the National High-throughput Sequencing Centre, University of Copenhagen. Sequences are deposited in the DNA Data Bank of Japan (DDBJ) Sequence Read Archive under accession number DRA006408.

The sequence reads were demultiplexed in QIIME (Caporaso et al., 2010), and the subsequent data process was performed in Mothur v. 1.34.2 58 as described earlier (Bentzon-Tilia et al., 2015; Shiozaki et al., 2017). On average 22,009 sequences per sample (min = 902, max = 48,345) were included in downstream analyses. The sequences were clustered at 97% nucleotide similarity and representative sequences from the most abundant operational taxonomic units (OTUs) were identified by Basic Local Alignment Search Tool (BLAST) searches against the National Center for Biotechnology Information (NCBI) nucleotide database.

2.7. Quantitative Polymerase Chain Reaction

Quantitative polymerase chain reaction (qPCR) analysis was applied to all DNA samples collected. Seven *nifH* phylotypes, which were considered to be the major diazotrophs in these tropical and subtropical oligotrophic waters, were quantified by qPCR: *Trichodesmium*, *Crocosphaera*, UCYN-A1, UCYN-C, *Richelia* associated with *Rhizosolenia* (RR) or *Hemiaulus* (HR), and a gammaproteobacterium (γ-24774A11). We used previously designed primer and probe sets (Table S1; Foster et al., 2007; Langlois et al., 2008; Moisander et al., 2010; Shiozaki, Chen, et al., 2014) in a LightCycler 480 System (Roche Applied Science, Germany) as described elsewhere (Shiozaki et al., 2015). All qPCR reactions were run in triplicate. Detected qPCR reaction efficiencies were between 86.6% and 108.7%. No signals were detected in negative controls. The detection limit was 75 copies L^{-1} seawater.

2.8. Satellite Data Analysis

Daily Moderate Resolution Imaging Spectroradiometer-Aqua (MODIS) level-3 chl *a* (mg m^{-3}) with 4-km resolution were obtained from NASA Goddard Space Flight Center (https://oceancolor.gsfc.nasa.gov/) for the period from 1 month before the ship observation to 5 days after in the western and eastern South Pacific. Chl *a* at each station was spatially averaged at a 2° × 2° resolution.

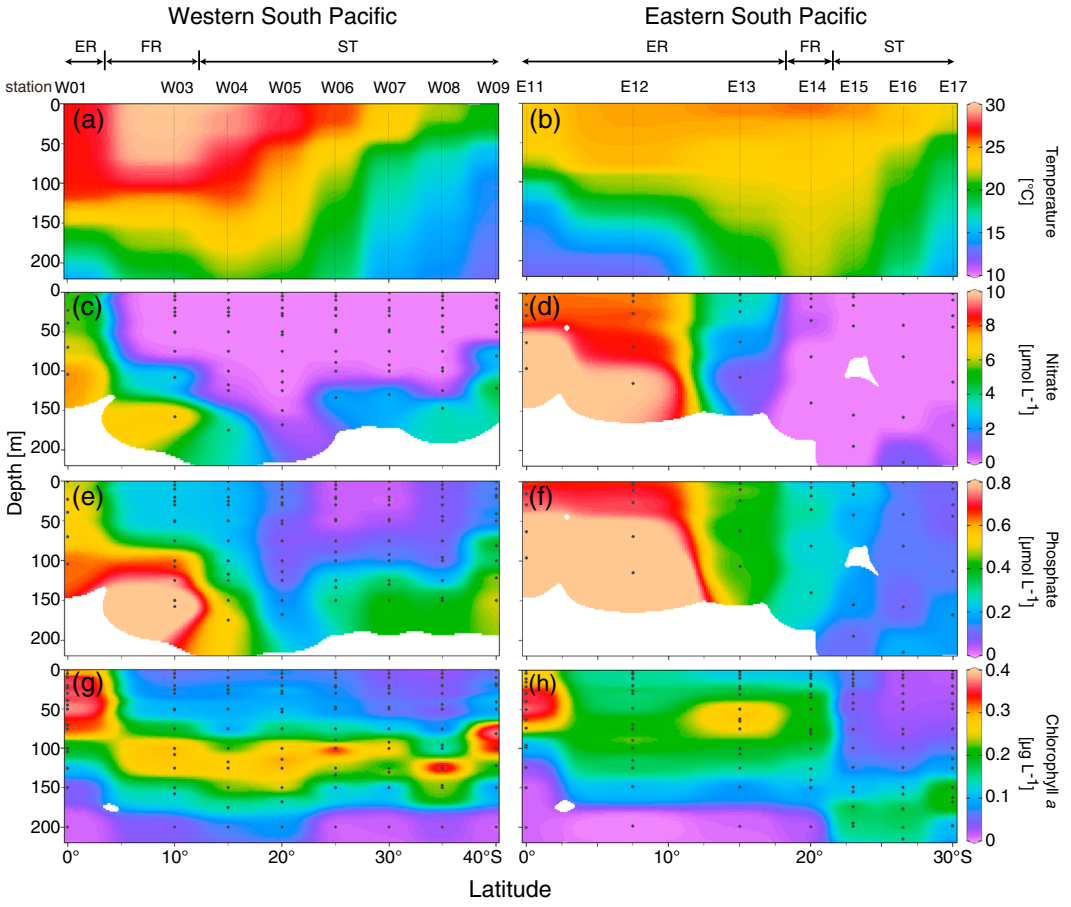

Figure 2. Latitudinal vertical distributions of (a, b) temperature, (c, d) nitrate, (e, f) phosphate, and (g, h) chlorophyll *a* along the transect in the western and eastern South Pacific. The environmental variables at St. E18–E22 in the eastern South Pacific present in Figure S1 and Data Set 1. ER, FR, and ST denote equatorial upwelling region, frontal region, and subtropical oligotrophic region, respectively.

3. Results

3.1. Environmental Conditions

Sea surface temperature (SST) ranged from 19.8 to 30.0°C and from 21.8 to 25.9°C in the western and eastern South Pacific, respectively (Figures 2a, 2b, and S1a). Based on observed surface (0 m) nitrate concentrations (Figures 2c, 2d, and S1b and Table 1), we classify our sampling stations into oceanic regions with differing trophic statuses: equatorial upwelling region (ER; $>$ 1000 nM), frontal region (FR; 10–1,000 nM), and subtropical oligotrophic region (ST; $<$ 10 nM). Nitracline depths (defined as the upper end concentration of the nitrate slope of 100 nM), were shallower in the western ST (44–127 m) than in the eastern ST ($>$116 m).

DON concentrations in surface waters (0 m) were high both in the ER and FR but decreased toward the ST. In the western ST, DON concentrations ranged 3.62–5.44 and 3.74–6.55 μM in the western and eastern ST, respectively while the surface inorganic nitrogenous nutrients including nitrate and ammonium were depleted both in the western and eastern ST (Table 1). Surface (0 m) phosphate concentrations showed large variation in the western (26–208 nM) and eastern (101–289 nM) parts of the ST (Figures 2e, 2f, and S1c and Table 1). The lowest phosphate concentration was observed at the 25% light depth of St. W07 (25 nM) in the western ST and at the 10% light depth of E17 (92 nM) in the eastern ST. Dissolved iron concentrations near the surface ranged from 0.06 to 0.36 nM and from 0.08 to 0.23 nM in the western and eastern ST, respectively (Table 1).

We used ocean color satellite observations to test how our own field measurements of chl *a* concentrations could be classified relative to longer-term dynamics of productivity in the area. Satellite-derived chl *a*

Table 1
Summary of Environmental Variables, Biological Production, and Vertical NO_3^- Flux in the Western and Eastern South Pacific

Station	Region[a]	NO_3^-[b] (nM)	NH_4^+[b] (nM)	DON[b] (μM)	PO_4^{3-}[b] (nM)	DFe[c] (nM)	1% (0.1%) Light depth (m)	N_2 fixation[d] (μmol N m^{-2} day^{-1})	NO_3^- assimilation[d] (μmol N m^{-2} day^{-1})	Vertical NO_3^- flux (μmol N m^{-2} day^{-1})	Primary production[d] (mmol C m^{-2} day^{-1})	N_2 fix/PP ratio[e] (%)
W01	ER	5530	43	5.30	495	0.23	70 (104)	n.d.	8750	30.1	35.2	0
W03	FR	12	-	6.03	235	0.18	108 (158)	n.d.	497	63.4	9.76	0
W04	ST	8	<4	5.44	208	0.36	117 (175)	n.d.	156	58.6	12.7	0
W05	ST	3	<4	4.90	127	0.29	114 (168)	131	41.4	10.6	32.2	2.69
W06	ST	<3	<4	4.79	26	0.19	89 (134)	209	42.4	49.9	23.3	5.91
W07	ST	<3	<4	4.20	30	0.06	92 (130)	93.4	47.9	63.2	11.9	5.18
W08	ST	<3	5	3.62	75	0.24	96 (147)	88.0	68.2	70.5	13.0	4.47
W09	ST	4	9	5.36	150	0.36	81 (122)	17.4	535	54.3	14.0	0.82
E11	ER	7890	-	6.81	650	0.02	63 (96)	73.1	6000	103	74.2	0.65
E12	ER	7880	-	7.70	613	0.10	69 (115)	n.d.	588	32.3	22.7	0
E13	ER	3500	-	6.17	380	0.30	62 (107)	19.7	664	—[f]	14.5	0.90
E14	FR	209	-	7.48	196	0.11	81 (140)	39.5	325	—[f]	13.7	1.90
E15	ST	4	17	6.55	227	0.20	155 (195)	453	124	0.07	7.35	40.7
E16	ST	3	<6	4.25	121	0.11	158 (215)	493	160	17.5	9.07	35.9
E17	ST	<3	<6	4.20	101	0.23	113 (168)	373	164	5.41	6.21	39.6
E18	ST	<3	7	4.26	136	0.08	135 (195)	359	57.2	25.7	9.26	25.6
E19	ST	3	14	3.74	153	0.14	110 (171)	284	139	25.8	11.3	16.6
E21	ST	5	<6	4.37	289	0.18	145 (205)	51.4	222	1.00	5.12	6.62
E22	FR	17	36	5.38	358	0.11	120 (177)	66.1	202	13.3	23.0	1.90

Note. DON = dissolved organic nitrogen; DFe = dissolved iron.
[a]Based on geographical location and nitrate concentrations in surface waters, three oceanic regions were classified, namely, the equatorial upwelling region (ER; $NO_3^- > 1{,}000$ nM), the frontal region (FR; 10 nM $< NO_3^- <$ 1,000 nM), and the subtropical oligotrophic region (ST; $NO_3^- < 10$ nM). [b]Surface data. [c]Data at 10 m and the 25% light depth in the western and eastern South Pacific, respectively. [d]Depth-integrated production from the surface to the 1% light depth. [e]N_2 fix/PP ratio = N_2 fixation × 6.6/primary production × 100, 6.6 is the Redfield ratio. [f]Nitrate concentration was higher in surface than at depth.

concentrations at each station of ST changed less than 20% within 1 month around our sampling dates, except at W09 and E15, which were located close to high-chl *a* waters (Figure S2 and S3). In the western ST, the satellite-derived chl *a* was increased at St. W06 (monthly average ± standard deviation, 0.048 ± 0.008 mg m^{-3}) compared to the neighboring stations (W05, 0.032 ± 0.006 mg m^{-3}; W07, 0.040 ± 0.003 mg m^{-3}) for a month (Figure S2). During the cruise periods, surface chl *a* in the western ST was significantly higher than in the eastern ST (Figures 2g, 2h, and S1d). At St. W04–W07, the average total cell volume of *Prochlorococcus* per surface sample was about fivefold higher than in the eastern ST (Figure 3). Since average total cell volume did not differ for eukaryotic phytoplankton between the western and the eastern ST, the high abundances of *Prochlorococcus* was largely responsible for the higher chl *a* in the western ST.

3.2. Nitrate Assimilation, Vertical Nitrate Flux, Dinitrogen Fixation, and Primary Production

Maximum rates of nitrate assimilation were always observed at the surface in both the western and eastern ER (Figures 4a, 4b, and S1e). Nitrate assimilation integrated to the 1% light depth was 8,750 μmol N m^{-2} day^{-1} (St. W01) in the western ER and 588–6,000 μmol N m^{-2} day^{-1} (St. E11–E13) in the eastern ER (Table 1). In the FR, nitrate assimilation was highest at the surface and/or near the bottom of the euphotic zone, suggesting that nitrate supplied to the euphotic zone via advection and diffusion from below was immediately consumed by microbes. In the ST region, maximum nitrate assimilation rates always occurred near the bottom of the euphotic zone. The depth-integrated nitrate assimilation tended to decreased toward the middle of the ST (west: W05–W07, east: E16–E19). Nitrate assimilation in the western ST (41.4–535 μmol N m^{-2} day^{-1}) was not significantly different from rates in the eastern ST (57.2–222 μmol N m^{-2} day^{-1}; $p > 0.05$, *t* test). Vertical nitrate flux varied 10.6–70.5 μmol N m^{-2} day^{-1} in the western ST and 0.07–25.8 μmol N m^{-2} day^{-1} in the eastern ST (Table 1).

Dinitrogen fixation was quantifiable only in the ST in the western South Pacific, while it was detected at all stations except St. E12 in the eastern South Pacific (Figures 4c, 4d, and S1f). Highest volumetric dinitrogen fixation occurred between the surface and the 10% light depth in the western (0.30–4.98 nmol N L^{-1}

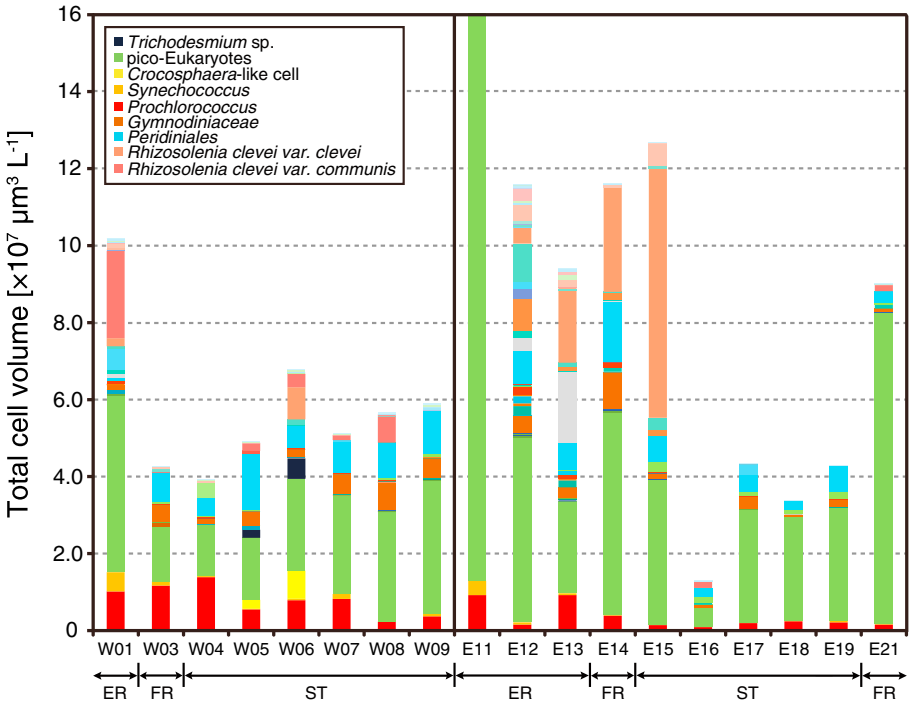

Figure 3. Total cell volume of phytoplankton at the surface. Representative groups are written in the legend. The total cell volume of each group is written in Data Set 2 in the supporting information. ER, FR, and ST denote equatorial upwelling region, frontal region, and subtropical oligotrophic region, respectively.

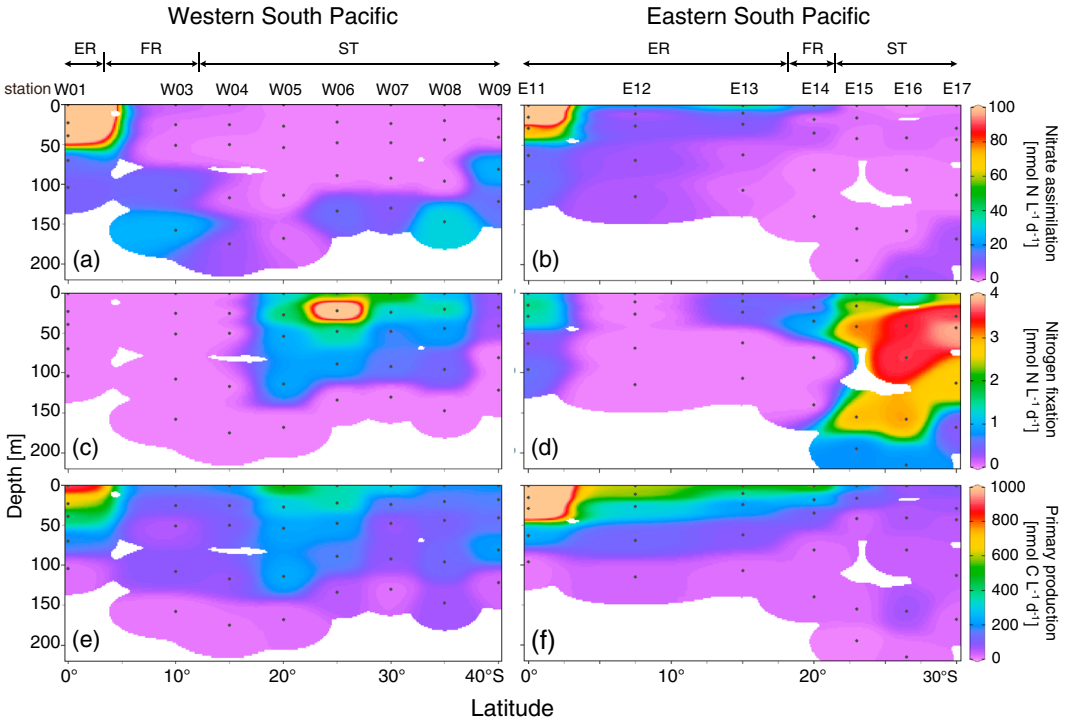

Figure 4. Latitudinal vertical distributions of (a, b) nitrate assimilation, (c,d) nitrogen fixation, and (e,f) primary production along transects in the western and eastern South Pacific. The activities at St. E18–E22 in the eastern South Pacific are presented in Figure S1 and Data Set 1.

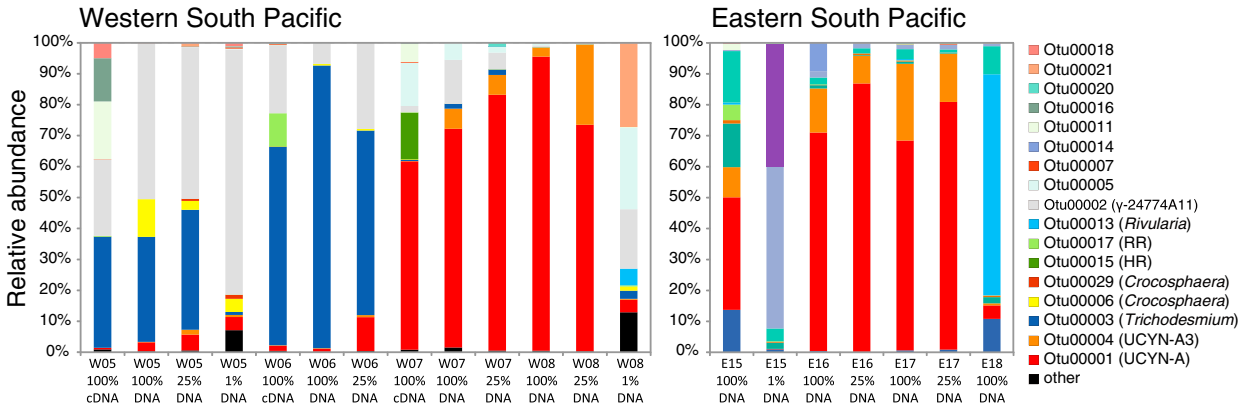

Figure 5. Composition of the total (DNA) and the active (cDNA) diazotroph community in waters of the analyzed stations, as inferred from Illumina sequencing of nitrogenase gene (*nifH*) amplicons. For the taxonomic affiliations of the operational taxonomic units, see Figure S3. RR and HR denote *Richelia* associated with *Rhizosolenia* and with *Hemiaulus*, respectively.

day^{-1}) and eastern (0.40–3.90 nmol N L^{-1} day^{-1}) ST, with no significant difference between these ST subregions ($p > 0.05$, *t* test). Depth-integrated dinitrogen fixation exceeded nitrate assimilation at St. W05–W08 in the western ST and at E15–E19 in the eastern ST. Furthermore, except at St. W09, dinitrogen fixation was higher than vertical nitrate flux in the ST.

Primary production was always highest at the surface both in the western and eastern South Pacific (Figures 4e, 4f, and S1g). In the western ST, primary production was elevated at St. W05 (32.2 mmol C m^{-2} day^{-1}) and W06 (23.3 mmol C m^{-2} day^{-1}) relative to the other stations. Primary production in the western ST (12.7–32.2 mmol C m^{-2} day^{-1}) was significantly higher than in the eastern ST (7.35–11.3 mmol C m^{-2} day^{-1}; $p < 0.05$, *t* test). The ratio of dinitrogen fixation to total nitrogen demand of primary production (= dinitrogen fixation × 6.6/primary production, where 6.6 is the Redfield ratio) was 0–5.91% (average: 3.18 ± 2.41%) in the western ST which was significantly lower than in the eastern ST (6.62–40.7%, average: 27.5 ± 13.8%; $p < 0.05$, *t* test; Table 1).

3.3. Diazotroph Community Composition

The *nifH* amplicons from DNA and RNA samples constituted 394 OTUs, of which the 17 most abundant accounted for >99% of all sequences. These were all affiliated with *nifH* Cluster I (Zehr et al., 2003). Seven OTUs clustered with cyanobacteria (Figure S4). Otu00001 was identical with UCYN-A1, and Otu00004 showed 98% nucleotide similarity to UCYN-A3. Otu00003 was identical with a *Trichodesmium*. Otu00006 and Otu00029 showed 100% and 94% nucleotide similarity to *Crocosphaera watsonii*, respectively. Otu00015 and Otu00017 showed 96% and 99% nucleotide similarity to *Richelia* associated with *Hemiaulus* (HR) and *Rhizosolenia* (RR), respectively. Otu00013 showed 96% nucleotide similarity with *Rivularia*. Among the noncyanobacterial OTUs, Otu00002 showed 98% nucleotide similarity to gammaproteobacteria γ-2477A11, and Otu00014 was identical with BAL376 which was isolated from surface water in the Baltic Sea (Farnelid et al., 2014), while the rests did not match with known diazotrophs.

Overall, cyanobacteria dominated the diazotroph communities in the surface waters, whereas noncyanobacteria dominated in deeper waters both in the western and eastern ST (Figure 5). The dominant diazotroph in the surface water in the western ST was *Trichodesmium* at St. W05 and W06, and UCYN-A1 at St. W07 and W08. In the eastern ST, it was UCYN-A1 at St. E15, E16, and E17, and *Rivularia* at St. E18.

The qPCR results were consistent with the structure of the total and active diazotroph communities as inferred from DNA and RNA sequencing. In the western ST, all target *nifH* phylotypes except UCYN-C was detected. In the western ST, *Trichodesmium* showed elevated abundance at St. W05 and W06 and maxima at the 25% light depth of St. W05 (1.4×10^4 copies L^{-1}) and at the surface of St. W06 (1.8×10^5 copies L^{-1}; Figure 6a). At the same stations, *Crocosphaera* showed maxima at the surface of St. W05 (7.2×10^4 copies L^{-1}) and at the 25% light depth of St. W06 (1.6×10^5 copies L^{-1}; Figure 6c). Abundance of UCYN-A1 was low near the surface at St. W05 and W06 and highest at the 10% light depth (9.1×10^4 and 4.9×10^5 copies L^{-1},

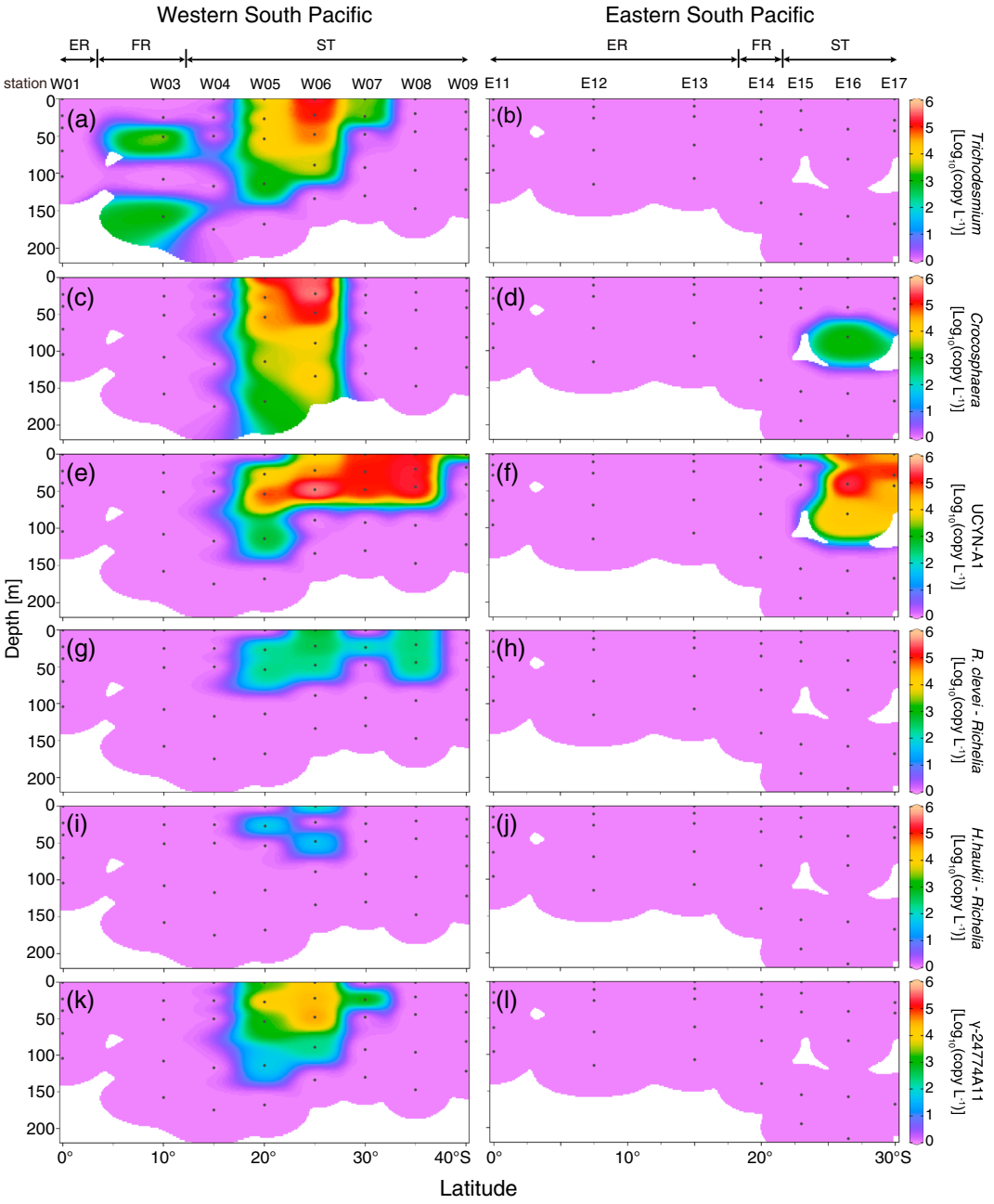

Figure 6. Latitudinal vertical distributions of (a, b) *Trichodesmium*, (c, d) *Crocosphaera*, (e, f) UCYN-A1, (g, h) *R. clevei-Richelia*, (i, j) *H. haukii-Richelia*, and (k, l) γ-24774A11 along the transect in the western and eastern South Pacific. The *nifH* abundances at St. E18–E22 in the eastern South Pacific are presented in Figure S1 and Data Set 1.

respectively) but homogenously distributed from the surface to the 10% light depth at St. W07 and W08 (maximum: 1.3×10^5 and 2.0×10^5 copies L^{-1}, respectively; Figure 6e). Although RR and HR sequences were found from St. W05 to W08 and from St. W05 to W06, respectively, their abundances were low ($<1.0 \times 10^3$ copies L^{-1}; Figures 6g and 6i). Abundance of γ-2477A11 (1.8×10^4 copies L^{-1}) was as high as *Trichodesmium* at St. W05 (Figure 6k). In the eastern ST, the only quantifiable *nifH* phylotypes were UCYN-A1 and *Crocosphaera* (Figures 6d, 6f, and S1 and Data Set S1). *Crocosphaera* occurred only at the 10% light depth at St. E16. UCYN-A1 was exclusively found between St. E16 and E18, with maximum abundances of 2.3×10^5 copies L^{-1} at 25% light depth at St. E16.

4. Discussion

Although a number of studies on dinitrogen fixation have been conducted in the South Pacific Ocean (e.g., Bonnet et al., 2015; Bonnet, Berthelot, Turk-Kubo, Fawcett, et al., 2016; Bonnet, Berthelot, Turk-Kubo, Cornet-Barthaux, et al., 2016, 2017; Dekaezemacker et al., 2013; Garcia et al., 2007; Halm et al., 2012; Shiozaki, Kodama, & Furuya, 2014), only few have tried to classify the magnitude of dinitrogen fixation in the context of total observed new production (Caffin et al., 2018; Knapp, Casciotti, et al., 2016, 2018; Raimbault & Garcia, 2008) and to relate observed rates to local primary production. Caffin et al. (2018) recently examined the contribution of dinitrogen fixation to new production both in the western and eastern subtropical South Pacific and reported that dinitrogen fixation always exceeded vertical nitrate flux. On the other hand, on the same cruise, Knapp et al. (2018) concluded that dinitrogen fixation was a minor contributor to export production in the eastern South Pacific while it was major in the west according to the $\delta^{15}N$ values in sinking particles. The difference between the two studies may originate from the different time scales affecting new and export production. Our data show from various angles that dinitrogen fixation was a major source of new nitrogen at most stations in the ST of the western and eastern South Pacific. In addition, satellite analysis suggested that biological production was stable from around 1 month before the observation both in the western and eastern subtropical stations. However, it should be noted that our cruise observations, especially rate measurements, only provide snapshots in time and may not necessarily scale to longer time periods.

In our data set, the effect of diazotrophs activities on primary production appeared highly dependent on whether the diazotroph community was dominated by *Trichodesmium* (high primary production) or unicellular diazotrophs including photoheterotrophs and heterotrophs (low primary production).

4.1. Nitrate-Based New Production and Phytoplankton Community in the Western and Eastern Subtropical South Pacific

Nitrate assimilation rate in the tropical and subtropical ocean is highly dependent on nitrate availability (Aufdenkampe et al., 2001; Shiozaki et al., 2009, 2016). The depth-integrated nitrate assimilation decreased toward the middle of the ST region both in western and eastern South Pacific, likely due to limited vertical and horizontal nitrate supply, and was comparable to rates reported for the subtropical North Pacific (Kanda, 2008; Shiozaki et al., 2009, 2017). Although surface chl *a* concentration in the western ST was significantly higher than in the eastern ST, nitrate assimilation at the surface was similar in the western ST (0.32–2.12 nmol N L^{-1} day^{-1}) and in the eastern ST (0.31–5.53 nmol N L^{-1} day^{-1}). Microscopy and flow cytometry indicated that the higher chl *a* in the western ST was caused by higher abundance of *Prochlorococcus*. Some lineages of *Prochlorococcus* may utilize nitrate (Berube et al., 2016; Martiny et al., 2009), but *Prochlorococcus* generally use regenerated nutrient such as ammonium as the nitrogen source (Fawcett et al., 2011; Moore et al., 2002). This suggests that the contribution of *Prochlorococcus* to nitrate assimilation was likely low. Therefore, nitrate assimilation would be mainly carried out by phytoplankton other than *Prochlorococcus*.

4.2. Nitrogen Fixation and Microbial Community in the Western and Eastern Subtropical South Pacific

Important factors regulating dinitrogen fixation include phosphorus and iron (Kustka et al., 2002; Mills et al., 2004; Sañudo-Wilhelmy et al., 2001). Phosphorus limitation of dinitrogen fixation generally occurs in phosphate-depleted waters ($<$ 1 nM; Sañudo-Wilhelmy et al., 2001; Wu et al., 2000). Since we did not encounter such low-phosphate waters, phosphorus limitation of diazotrophy did not likely occur during our cruises. The minimum phosphate concentration was higher in the eastern ST than in the western ST, and the similar concentrations and patterns have been observed previously (Moutin et al., 2008). Interestingly, surface phosphate concentration tended to be low at stations where dinitrogen fixation was high both in the western and eastern ST, probably due to uptake by diazotrophs. Dissolved iron concentrations near surface in the eastern ST (0.08–0.23 nM) were similar to those previously reported from the same region (0.09–0.20 nM; Blain et al., 2008). In the western ST, dissolved iron concentrations near surface (0.06–0.36 nM) were in the lower end of concentrations reported previously from this region ($<$0.06–1.0 nM; Campbell et al., 2005). The low concentrations of dissolved iron may indicate that dinitrogen fixation was limited by iron. Concentrations of dissolved iron near the surface were similar in the western and eastern ST, which may contribute to the fairly similar rates of dinitrogen fixation, although it should be noted that we may have

underestimated dinitrogen fixation in the eastern ST because it was determined by the $^{15}N_2$ gas bubble method (Mohr et al., 2010).

Dinitrogen fixation rates in the western ST were in the lower end of values previously reported for the region (2–5,449 μmol N m^{-2} day^{-1}; Bonnet et al., 2015, 2017; Garcia et al., 2007; Shiozaki, Kodama, & Furuya, 2014). Abundances of *Trichodesmium* and *Crocosphaera* were elevated at the high primary production and high dinitrogen fixation sites in the western ST (St. W05 and W06). Further, sequencing of *nifH* gene transcripts suggested that *Trichodesmium* was a major contributor to dinitrogen fixation at these stations during daytime. On the other hand, UCYN-A1 abundances corresponded well with the elevated dinitrogen fixation rates at St. W07 and W08.

Dinitrogen fixation in the eastern ST was up to 3.59 nmol N L^{-1} day^{-1} and thus higher than earlier reported values (maximum: <3 nmol N L^{-1} day^{-1}; Dekaezemacker et al., 2013; Halm et al., 2012; Raimbault & Garcia, 2008). Consistent with earlier studies in the eastern ST (Bonnet et al., 2008; Halm et al., 2012; Turk-Kubo et al., 2014), major *nifH* phylotypes were UCYN-A1 and heterotrophic bacteria; however, abundances of UCYN-A1 in our study (up to 2.4×10^5 copies L^{-1}) were much higher than earlier reports in the middle of eastern ST ($<1.0 \times 10^3$ copies L^{-1}; Bonnet et al., 2008; Halm et al., 2012; Turk-Kubo et al., 2014). Dinitrogen fixation was especially higher in the waters with abundant UCYN-A1. The Miseq and qPCR analyses indicate that UCYN-A1 dominated the diazotroph communities at the 25% and 100% light depths at St. E16 and E17. In these waters, cell specific dinitrogen fixation by UCYN-A1 was calculated to be 14–77 fmol $cell^{-1}$ day^{-1}, which is within the range of that estimated from single-cell analyses by Nano-SIMS (12–220 fmol $cell^{-1}$ day^{-1}; Martínez-Pérez et al., 2016). Hence, we think that is conceivable that UCYN-A1 was responsible for the irregularly high dinitrogen fixation measured in the eastern ST.

Chl *a* was not elevated at St. E16 and E17 compared to the neighboring stations (Figure 2), and also not from around 1 month before the cruise according to satellite observation (Figure S2). This could be due to the poor pigmentation of UCYN-A (Zehr et al., 2008), although not much is known about the pigmentation signal of the host organism. Temperature is known to be related with the abundance of UCYN-A1 (Moisander et al., 2010), and the SST at St. E16 and E17 (24.4 and 22.7°C) was similar with that at St. W07 and W08 (24.0 and 21.9°C) in the western ST where high abundance of UCYN-A1 occurred. In previous studies in the eastern ST, the SST at some stations was in a similar range with the present study; however, UCYN-A1 abundance was low (Bonnet et al., 2008; Halm et al., 2012; Turk-Kubo et al., 2014). Therefore, observed high abundance of UCYN-A1 in the eastern ST could not be simply explained by the temperature, but we were unable to identify other factors influencing its abundance. Active dinitrogen fixation (>2 nmol N L^{-1} day^{-1}) was also observed in deeper waters void of UCYN-A1 but where the diazotroph community was dominated by heterotrophic bacteria (e.g., at the 1% light depth of St. E15). Many *nifH* sequences clustering with *Rivularia* were found at St. E18, but the low dinitrogen fixation (0.86 nmol N L^{-1} day^{-1}) suggests that these were not particularly active in dinitrogen fixation. Consequently, the dinitrogen fixation in the eastern ST must have been performed mainly by UCYN-A1 and heterotrophic bacteria.

4.3. Linkage Between Dinitrogen Fixation and Primary Production

Primary production and dinitrogen fixation were relatively high at St. W05 and W06 in the western ST, and since dinitrogen fixation was higher than nitrate assimilation and vertical nitrate flux, we infer that the primary producers benefited from the substantial nitrogen input from diazotrophy during our period of sampling. Although depth-integrated dinitrogen fixation at some stations in the eastern ST was higher than that at St. W05 and W06 in the western ST, there was no corresponding elevation of primary production. Similarly high rates of dinitrogen fixation enhanced primary production north of the Hawaii Islands (Church et al., 2009) and in the subtropical North Atlantic (Mouriño-Carballido et al., 2011) but not in the subtropical central North Pacific (Shiozaki et al., 2017). In regions where high primary production and high dinitrogen fixation was found, abundances of *Trichodesmium* were usually relatively high (Church et al., 2009; Mouriño-Carballido et al., 2011; this study). *Trichodesmium* is known to release large shares (up to 90%) of their fixed dinitrogen as DIN and DON (Glibert & Bronk, 1994; Mulholland & Bernhardt, 2005), thereby stimulating growth of co-occurring nondiazotroph plankton (Bonnet, Berthelot, Turk-Kubo, Cornet-Barthaux, et al., 2016; Chen et al., 2011). The released nitrogen is likely immediately consumed by surrounding planktonic communities, and indeed, we did not observed DIN and DON accumulation in the western ST. The higher

abundances of *Prochlorococcus* mirrored the scenario of a system mainly driven by regenerated nitrogen. While regenerated production supports the majority of primary production in the oligotrophic ocean, it generally does not raise the baseline of primary production (Dugdale & Goering, 1967; Falkowski et al., 2003); however, new nitrogen released from diazotrophs could increase primary production. Carbon fixation by *Trichodesmium* itself likely also contributed to the high primary production at St. W05 and W06. The cell volume of *Trichodesmium* accounted for 4.3% and 7.8% of total phytoplankton cell volume at St. W05 and W06, respectively (Figure 3). Cell specific carbon fixation rate by *Trichodesmium* is known to vary significantly with light intensity (0.57–2.12 pmol C cell^{-1} day^{-1}; Kranz et al., 2010). We calculated a conservative estimate of depth-integrated primary production by *Trichodesmium* by using the minimum cell specific rate. Estimates were 0.63 and 3.9 mmol C m^{-2} day^{-1} at St. W05 and W06, corresponding to 2.0% and 12% of total primary production, respectively. This estimate suggests that carbon fixation by *Trichodesmium* itself contributed significantly to primary production, especially at St. W06.

Crocosphaera abundances were also relatively high at St. W05 and W06 and were similar with *Trichodesmium* abundances determined by qPCR analysis. However, their dinitrogen fixation and carbon fixation might not contribute significantly to primary production, since the transfer efficiency of fixed dinitrogen from *Crocosphaera* to nondiazotrophic plankton is apparently only half of that of *Trichodesmium* (Berthelot et al., 2016), and carbon fixation rates per *Crocosphaera* cell is less than one third of that of *Trichodesmium* cell (Berthelot, Bonnet, et al., 2015). A similar case was described for the central subtropical North Pacific, where the diazotroph community was dominated by *Crocosphaera*. Here dinitrogen fixation (228 μmol N m^{-2} day^{-1}) was as high as in the middle of western ST, but primary production was not enhanced (Shiozaki et al., 2016, 2017).

In the eastern ST, the relatively low primary production together with dinitrogen fixation apparently mainly carried out by UCYN-A1 and heterotrophic bacteria suggest a relatively weak direct stimulation of primary production by diazotroph N inputs. UCYN-A1 lacks RuBisCO and thus do not perform carbon fixation and mainly function as *diazotroph organelles* within their autotroph, eukaryotic hosts (Bombar et al., 2014; Tripp et al., 2010). UCYN-A is known to efficiently channel fixed dinitrogen to its prymnesiophyte host (Martínez-Pérez et al., 2016). The fate of dinitrogen fixed by UCYN-A1 is not well understood, but in other symbioses, dinitrogen fixed by the diazotroph remains in the symbiosis and does not fuel surrounding planktonic communities (Berthelot, Moutin, et al., 2015).

The relationship between dinitrogen fixation by UCYN-A and heterotrophic bacteria and primary production deserves further scrutiny. Interestingly, the ratio of dinitrogen fixation to total nitrogen demand for primary production in the eastern ST (6.62–40.7%, Table 1) was in the range of previously reported values (6.15–60.1%) in the same region (Halm et al., 2012) but was mostly higher than the typical value of *f* ratio in the oligotrophic ocean (<20%; Falkowski et al., 2003). The high percentage in the eastern ST supports the notion that most of fixed dinitrogen by UCYN-A1 and heterotrophic bacteria did not immediately fuel growth of primary producers. Taken together, our results indicate that diazotroph community structure can be essential for the linkage between dinitrogen fixation and primary production. This is a clearly different scenario from the one of nitrate-based new production immediately fueling primary production: while the steering factors of nitrate-based new production are mainly physical (e.g., wind-induced vertical mixing and mesoscale eddies), the ones for new production due to diazotrophy are unequally more complex since they also include chemical and biological interactions resulting in different diazotroph communities present at a given location (Moisander et al., 2010; Sohm et al., 2011; Shiozaki, Ijichi, et al., 2014), with considerable effects on the magnitude of local primary production.

5. Conclusion

The present study shows that the stimulation of primary production by diazotrophs in the oligotrophic ocean would be highly dependent on the composition of the diazotroph communities. Dinitrogen fixation carried out by *Trichodesmium* likely stimulated primary production to a much larger extent than if the same fixation was carried out by unicellular organisms like UCYN-A, *Crocosphaera*, or heterotrophic bacteria. In addition, it is noteworthy that the carbon fixation by autotrophic diazotrophs themselves, especially *Trichodesmium* due to its higher cell specific carbon fixation rate, contribute to total primary production. Our conclusions are derived from field observations, and to be more solid, laboratory works using

isolated cultures, especially UCYN-A1 and heterotrophic bacteria, are required. If our hypothesis is valid, the links between local diazotroph N inputs and the carbon cycle are more complex and need to take contemporary diazotroph community composition into account. As mentioned, diazotroph community composition is highly dynamic and varies due to many factors. The link between diazotroph composition and the effect of diazotrophs activities on primary production, suggested by the present work, could have ample implications for our understanding of current controls on primary production in the vast regions of the global oligotrophic ocean.

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